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<b>(21) International Application Number:</b> PCT/GB97/01473 <b>(22) International Filing Date:</b> 30 May 1997 (30.05.97) <b>(30) Priority Data:</b> 9611420.2 31 May 1996 (31.05.96) GB <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF YORK [GB/GB]; Heslington, York YO1 5DD (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BOWLES, Dianna, Joy [GB/GB]; The University of York, Heslington, York YO1 5DD (GB). O'DONNELL, Philip, James [GB/GB]; The University of York, Heslington, York YO1 5DD (GB). ROBERTS, Michael, Richard [GB/GB]; The University of York, Heslington, York YO1 5DD (GB). CALVERT, Caroline, Mary [GB/GB]; The University of York, Heslington, York YO1 5DD (GB). <b>(74) Agents:</b> CHAPMAN, Paul, William et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> USE OF A NOVEL GLUCOSYL TRANSFERASE  <b>(57) Abstract</b>  This invention relates to methods for inducing plant defence and resistance responses as well as regulating plant developmental events in monocots and dicots by modifying a gene encoding for a glucosyl transferase (TWI1) isolated from wounded tomatoes.		

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USE OF A NOVEL GLUCOSYL TRANSFERASE

This invention relates to a method for inducing plant defence and resistance responses and regulating plant developmental events. More particularly, this invention relates to methods for inducing the production of plant defence proteins such as pathogenesis-related (PR) proteins and proteinase inhibitor (pin) proteins and to methods for regulating resistance and acquired resistance to predators, insects, bacteria, fungi and viruses in plants, through manipulating levels of the plant hormones: salicylic acid, jasmonic acid, cytokinins and ethylene, and to methods for regulating developmental events that depend on these hormones, particularly, plant growth, reproduction and senescence.

Adaptation of a plant to its environment is brought about by recognition and response to external stimuli which cause changes in cellular activity. A chain of events link the initial recognition of the stimulus to changes in cells of the plant that ultimately lead to adaptation. These events constitute a signal transduction pathway, in which sequential molecular interactions transduce (lead) the signal from its perception through to the end-effects caused. Plants respond to a vast range of environmental stimuli that include, for example: changes in their growing conditions (light, heat, cold, drought, water-logging etc); mechanical damage leading to injury, and challenge by pests and pathogens (herbivores, insects, fungi, bacteria, viruses etc). These stimuli lead to cellular events at the site(s) of perception, but also can trigger long-range events throughout the plant,

leading to systemic changes. Thus, for example, in response to wounding and to pest/pathogen challenge, there are local and systemic events induced with signal transduction pathways occurring at the local site, systemic signal(s) communicating the local events around the plant, and signal transduction pathways occurring in distant cells that are responding to the systemic signal(s). Networking and cross-talk between intra- and inter-cellular signal transduction pathways is recognised to be an important means through which the plant integrates all of the information received from the environment.

Plant hormones play a central role in these induced responses to environmental stimuli, since they act as the intermediate molecular signals which trigger the transduction pathways leading from the external change(s) in the environment to the internal end-effect(s) within the plant. For example, in a variety of plant species, jasmonic acid is known to accumulate transiently during the wound response and has been implicated in transduction events linking mechanical injury to activation of wound-responsive genes. Another example is during the interactions of plants with pests and pathogens, when salicylic acid is known to increase in quantity (together with its precursor, benzoic acid and its volatile form, methyl salicylate) and is considered to be a central regulator of local and systemic acquired resistance and the activation of defence-related genes associated with resistance.

Whilst salicylic acid is a positive regulator of these induced resistance responses, there is evidence to show that the hormone is also a negative regulator of the

wound response, such that if a plant is pre-treated with aspirin or salicylic acid, wound-responsive genes dependent on jasmonic acid for their expression are not induced [1,2,3,4]. This suggests there is  
5 communication between the signalling pathways induced by mechanical injury and induced by pests/pathogens, such that they do not occur simultaneously.

Senescence is the natural process which normally leads  
10 to cell death, either in a selected population of cells such as abscission cells or in whole organs such as flowers, leaves and fruits. This process of senescence can be developmentally regulated, such as, for example, in the ripening process, wilting and fading of flowers,  
15 yellowing and abscission of leaves. Alternatively, senescence and cell death can be induced by trauma, such as caused by, for example, chemicals, temperature extreme, pest and pathogen damage, disease or mechanical wounding.

20 The level of an active plant hormone in plant cells and tissues at any one time is dependent on the relative rates of its synthesis and degradation, the rates of transport to or from the cells/tissue, and the relative  
25 rates of its conversion to and from inactive metabolite(s). For plant hormones, this conversion to an inactive metabolite can involve the conjugation of the free active form of the hormone to a polar molecular species, such as a sugar, amino acid or  
30 peptide. Endogenous hormones made by the plant, and exogenous hormones applied to and taken up by the plant are subject to conjugation in this manner. When large quantities of exogenous hormones are applied to the plant the conjugation process has been likened to

detoxification since it effectively clears the active hormones from the system rapidly.

5 Since the process of conjugation is known to be reversible in plants, at least for some hormones, it provides a flexible mechanism for regulating the pool size of active hormone in the absence of synthesis and degradation. Also, since most plant hormones are  
10 either apolar or amphiphilic, their reversible conjugation to a polar molecule provides a useful mechanism for containing the hormone on one side or other of a membrane, such as in the apoplast, or in a particular compartment of the symplast.

15 Whilst many different conjugates of plant hormones have been identified, a commonly found conjugate is the glucoside, formed through the transfer of glucose from a sugar nucleotide donor to the hormone via a  $\beta$ , O-glycosidic linkage. The enzymes responsible are  $\beta$ -  
20 glucosyl transferases and the available evidence indicates that each transferase is highly specific for the particular hormone it conjugates. For example, the glucosyl transferase responsible for conjugation of the plant hormone, indole 3-acetic acid, has been  
25 identified and shown to be specific for the auxin substrate [5]. Similarly, the glucosylation of salicylic acid has also been investigated, and an enzyme activity identified in a variety of plant species has been shown to be specific for salicylic  
30 acid and the sugar nucleotide donor, UDP glucose [6].

Another important hormone is ethylene which is a gas under physiological conditions that influences a wide range of events in plants, including the regulation of

growth, cellular differentiation and developmental processes. In particular, ethylene is the key regulator of senescence, which as stated above, is a genetically controlled process of degeneration normally leading to cell death and which occurs in specific cell-types and in whole organs such as flowers, leaves and fruits. The effects of ethylene on developmental processes are of considerable commercial importance. The effects can occur at low concentrations, whether the gas is produced by the plant itself or applied exogenously to the plant. Ethylene is also involved in defence/stress and resistance responses, such as directing how the plant combats challenges from pests and pathogens, and during the consequences of abiotic stimuli, for example, mechanical injury and water-logging. In these defence/stress and resistance responses, ethylene has a direct effect on the activation of specific genes, as well as a role in inducing cell death associated with hypersensitive responses.

Generally, ethylene is maintained at very low levels in plant tissue, but production can be rapid and massive during the senescence process, or during stress/trauma caused by biotic and abiotic stimuli. During the degenerative process of senescence, ethylene synthesis is regulated by positive feedback, such that one action of the ethylene produced is the up-regulation of the synthetic machinery and thus further production of more ethylene leading to an autocatalytic avalanche of increased levels of the hormone. In contrast, during stress/trauma, the "wound" or "stress" ethylene produced is regulated by negative feedback, leading to a hormone transient. Ethylene has been

shown unequivocally to be a requirement for the developmental senescence process. The role of "wound" ethylene is less defined.

- 5 The ethylene signal transduction pathway is the most characterised of all plant hormones to date, with the identification of genes encoding a receptor, a negative regulator protein and a number of proteins implicated genetically in downstream events [7]. In contrast,
- 10 very little is known of the regulation or the mechanism(s) by which ethylene levels rise in response to the environmental stimuli and to pest and pathogen attack.
- 15 Since plants have evolved inducible mechanisms of defence that respond to attack by pests and pathogens, there is considerable commercial interest in identifying methods of induction which will protect and even enhance the natural resistance of the crop plant.
- 20 This is particularly relevant when the only agrochemicals available are hazardous both to the environment and the consumer. Often, during the natural course of a defence response to pest and pathogen challenge, a broad spectrum of defence-related
- 25 genes and physiological events are induced in parallel and their success at conferring resistance arises from the multiplicity of their actions. Long-term efficacy of this strategy is much greater than that achieved by genetically modifying crop plants with single defence-
- 30 related genes. This is because in field situations the alteration or insertion of a single defence-related gene can be overcome by pests and pathogens rapidly evolving and adapting to the single gene change. In addition, decreased crop yields and decreased product

quality are features commonly encountered in resistant cultivars. Thus, there exists a strong requirement for new materials and processes to improve the resistance of plants under attack by pests and pathogens. This would preferably be through the induction of the plant's own defence systems.

There is also considerable commercial interest in identifying the molecular "switches" which respond to non-hazardous chemicals applied to the plant, and in turn, regulate developmental and defence responses, where and when applied. Whilst some inducible promoters have been found that are responsive to various chemicals, (e.g. PR gene promoters responsive to dichloroisonicotinic acid (DCINA) [8] which can be used to drive the expression of genes of interest), the range of applications envisaged could be increased dramatically if more promoters and more chemical inducers were identified. By way of background, DCINA is a widely used agrochemical which induces systemic acquired resistance (SAR) and is thought to act at a point downstream of salicylic acid in the transduction pathway leading to SAR gene expression.

For plant resistance and post-harvest protection of raw material quality, the speed of the defence response mounted by the plant cells/tissues, often determines the overall success-rate. Therefore, major interest lies in identifying rapidly responding promoters and, dependent on the application, those that are either capable of driving expression in a wide range of cell-types or those that are highly specific to a particular cell-type or tissue, for example, epidermal cells or leaves, but not stems, etc.

There is also major commercial interest in identifying ways in which senescence can either be controlled, prevented completely, the time-span of senescence regulated, or its occurrence induced only when  
5 required. Since senescence is intimately associated with ethylene, the problems of senescence are really problems of ethylene quantity and ethylene action. For example, in the post-harvest care of fruits, vegetables and flowers, cuts and bruising can stimulate ethylene  
10 production which in turn causes cell death in the traumatised tissues as well as affecting the adjacent fresh produce. This in turn leads to massive losses in the quality of these materials during transportation and storage. Traditional technologies addressing post-  
15 harvest issues have been tried for decades but suffer from problems of side-effects, toxicity, high costs and an inability to shut down completely ethylene synthesis [9].

20 In the present invention, a gene TWI1, whose existence had previously been established as merely being a "wound inducible" gene but whose true function, until now, was completely unknown, is disclosed. It is now known by way of our invention that the TWI1 gene codes  
25 for a glucosyl transferase (GTase) which regulates levels of a key signalling intermediate. Through detailed analyses of the expression patterns of TWI1 in tomato, this gene is believed to function in those signalling pathways leading to developmental and  
30 defence responses controlled by ethylene. Using transgenic plants with modified levels of TWI1 expression, we demonstrate a key role for this gene in plant responses to wounding and to pathogens.

A disclosure of a partial sequence on an EMBL database of a wounded tomato leaf library indicated that the TWI1 cDNA might possibly encode for a GTase. However, no indication was given as to the induction patterns of the TWI1 gene nor the possible function of its product. These factors were not deducible from the partial sequence disclosed, nor from the source of the mRNA from which the cDNA was derived. This invention, therefore, provides the first-ever correlation between GTase action and the regulation of ethylene, a common intermediate in a diverse process in plants.

Accordingly, the present invention provides a method of altering the signalling pathways of a plant involving salicylic acid, ethylene and jasmonic acid. The method comprises interfering with the normal functioning of the TWI1 gene encoding a GTase in plants. This is particularly useful in tomato plants, however, this invention would apply with similar advantage to other dicotyledonous or monocotyledonous plants (i.e. broad-leaved plant species as well as grasses and cereals). This invention is applicable to any horticultural or agricultural species, including those in which fruit-ripening and/or post-harvest storage are important considerations.

According to one aspect of this invention, there is provided a recombinant or isolated DNA molecule which encodes for a glucosyl transferase in plants. In preferred embodiments, the GTase gene (TWI1) comprises the nucleic acid sequence or at least portions (or fragments) of the nucleic acid sequence shown in FIGS 1 and 2. Also sequences having substantial sequence homology with the TWI1 gene of FIGS 1 and 2 are also

claimed in this invention. Moreover, sequences having substantial sequence homology with the amino acid sequence encoded by the TWI1 gene (FIG 3) are claimed as part of this invention.

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The term "portions" or "fragments" as used herein should be interpreted to mean that a sufficient number of nucleic acid or amino acid residues are present for the fragment to be useful (i.e. to act as or encode a GTase). Typically, at least four, five, six, up to 20 or more residues may be present in a fragment. Useful fragments include those which are the same as or similar or equivalent to those naturally produced by the TWI1 gene or its equivalent gene and enzyme in other plants, for example, as in FIGS 4 and 5 for rice and tobacco, respectively.

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As used in the present application, substantial sequence homology means close structural relationship between nucleotides or amino acids. For example, substantially homologous DNA sequences may be 60% homologous, preferably 80% and most preferably around 90 to 95% homologous, or more, and substantially homologous amino acid sequences may preferably be 35%, more preferably 50%, most preferably more than 50% homologous. Homology also includes a relationship wherein one or several subsequences of nucleotides or amino acids are missing, or subsequences with additional nucleotides or amino acids are interdispersed. When high degrees of sequence identity are present there may be relatively few differences in the amino acid sequences. Thus, for example, they may be less than 20, less than 10, or even less than 5 differences in amino acid sequences.

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The degree of amino acid sequence identity can be calculated, for example, using a program such as "BESTFIT" (Smith and Waterman, *Advances in Applied Mathematics*, pp. 482-489 (1981)) to find the best segment of similarity between any two sequences. The alignment is based on maximising the score achieved using a matrix of amino acid similarities, such as that described by Schwarz and Dayhof (*Atlas of protein Sequence and Structure*, Dayhof, M.O., pp. 353-358).

Using the TWI1 cDNA sequence as a probe to analyze expression of the GTase during a pathogen response in tomato, we observed that the gene is induced during gene-for-gene mediated resistance (R) response involving the Cf9 R gene to *Cladosporium fulvum*. Similarly, using a homologous GTase gene which we isolated from tobacco as a probe (FIG 4), we also found induction during gene-for-gene mediated R response involving the N gene to tobacco mosaic virus (TMV). In this latter system, induction of the GTase gene in response to TMV was causally dependent upon the elevation of salicylic acid. These data imply a role for the TWI1 gene product in salicylic acid-mediated pathogen responses. Thus, an object of this invention is the use of TWI1 gene in stimulating or improving pathogen related responses in plants, through induction of the GTase and thus effecting the levels of salicylic acid normally present in the plant (i.e. a wild-type plant).

In transgenic tobacco plants which constitutively express the GTase at a high level, we found that the formation of necrotic lesions and the induction of PR-gene expression in response to the bacterial elicitor,

harpin [10], is completely suppressed. In contrast, in plants in which GTase expression is repressed, the response to harpin is enhanced. These data show that the GTase gene product impacts directly on events at the local site of challenge with consequences on the process of acquired resistance. Importantly, the implication is that in transgenic plants expressing an antisense gene to the GTase, a hypersensitive response (HR) and acquired resistance to pathogen challenge may be enhanced.

In wound response, JA and ethylene are causally required for pin gene expression. The elevation of endogenous JA is very rapid and transient and is dependent on ethylene action [Bowles, et al, unpublished data]. Salicylic acid applied to plants prior to wounding inhibits this elevation in JA completely and also inhibits pin gene expression [Bowles, et al, unpublished data]. The wound induction of the GTase is also very rapid with a parallel time-course to the elevation in JA. In plants expressing the TWI1 antisense gene, wounding does not induce pin2 expression. Expression of the gene encoding ethylene-forming enzyme occurs as normal, but in contrast to wild-type plants, in the transgenic plants the down regulation no longer occurs. This provides further direct evidence for the role of the GTase gene product in the regulation of ethylene and ethylene-dependent responses.

GTase activity as it relates to ethylene has never previously been identified nor contemplated. This would not be an obvious nor routine method to follow despite existing literature on GTases. Further, the

developmental pattern of expression of any GTase is developmentally-regulated. This is demonstrated by the attached examples, wherein GTase is expressed at high levels in ethylene-mediated processes such as fruit-  
5 ripening and senescence. Moreover, since the gene was not previously available for relevant antisense experiments, the opportunity to analyze the effects of GTase down-regulation on ethylene did not exist prior to this invention.

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In this invention, we utilise antisense technology to demonstrate that down-regulation of GTase leads to prolonged levels of stress ethylene. Therefore, a further aspect of this invention is the use of the  
15 aforementioned GTase or any functional homologues thereto for use in down regulating GTase in a plant of interest.

Thus, according to a further embodiment of this  
20 invention, there is provided antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of the strand of DNA that is naturally transcribed in a gene encoding GTase. This involves the construction of transformation  
25 vectors possessing either the entire or partial coding sequence of the homologous GTase gene from the species to be transformed in the reverse orientation, under the transcriptional control of a constitutive promoter such as the Cauliflower Mosaic Virus 35S promoter and a  
30 transcription terminator sequence such as the *Agrobacterium tumefaciens* nos terminator. Also present in the vector, it is preferable, but not necessary, to include a plant selectable marker gene which enables a plant transformed with the TWI1 gene or a gene

substantially homologous thereto, to be distinguished from plants not so transformed. Such markers may include, for example, neomycin phosphotransferase II or hygromycin phosphotransferase. Typically these selective markers are for antibiotic or herbicide resistance. Vectors containing these sequences may either be broad host range binary vectors useful for *Agrobacterium*-mediated transformation such as those derived from pBin19 [13], or standard *E. coli* vectors useful for production of high levels of plasmid for transformation mediated by particle delivery. In addition to the tomato GTase antisense construct described in the examples below, we have also produced an antisense construct using sequences from the tobacco GTase gene of FIG. 4.

To achieve over-expression of the GTase, the coding sequence or portion of the coding sequence of the tomato TWI1 cDNA, or a coding sequence encoding an active homologous GTase isolated from any other organism or a nucleic acid sequence synthetically produced by means well-known to those skilled in the art, may be placed under the control of promoters activated specifically in the tissues of interest, these being preferably ripening fruit and senescing leaves or flowers, and followed by a transcription terminator sequence.

In the present invention, through use of Northern analyses, we show for the first time that a high expression of GTase exists in senescence and in ripened fruits. The implications of this are great, namely there may be an increased "need" for the GTase in ethylene-mediated events.

On an application point, it is easier to obtain the over-expression effects in any plant species since a heterologous gene will produce the same effects. For an antisense approach, the homologous gene is preferred  
5 and may be isolated for any commercially important plant or crop.

Another aspect of this invention is the use of a promoter comprising the 5' upstream region of the TWI1  
10 GTase gene. The promoter claimed under this invention or one similar (substantially homologous) to that of FIG 2 isolated from plants other than tomato would exhibit activation characteristics including rapid activation following mechanical wounding or pathogen  
15 attack, including activation by salicylic acid, various salicylic acid analogues thereof and the functionally-related compound, DCINA. We show that the wound induction of the TWI1 gene and the induction by the chemical elicitors is via two independent pathways.  
20 Also, promoters of homologous GTase genes from other plant species exhibiting similar activation characteristics in their respective species are also claimed by way of this invention.

25 Activation of the GTase at the appropriate times using promoters derived from other sources is also claimed within the scope of this invention ((e.g. at the onset of senescence; e.g. *Arabidopsis* SAG12 promoter [11]) or at the onset of ripening (e.g. tomato polygalacturonase  
30 promoter [12])).

The promoters in the present invention were isolated from clones obtained from a commercial genomic library of tomato by using TWI1 cDNA sequence as a probe.

Sequencing of such clones identified the GTase coding sequence, with the 5' upstream region to approximately 5 kilobases of the GTase transcription start sequence considered to be the promoter. Promoters of similar or substantially homologous GTase genes to the TWI1 gene of wounded tomato plants may be isolated from other genomic plant libraries or by utilising isolation techniques well-known to those skilled in the art, including, for example, the use of inverse polymerase chain reaction.

The promoter from the TWI1 gene is useful as a sequence capable of regulating the rapid accumulation of desirable gene products at sites of physical injury to a plant. Gene products considered desirable for such control include, but are not limited to: polypeptides with anti-microbial, antifungal, anti-insect etc activities, or polypeptides which have an activity which would protect the plant from further damage, for example, by altering cell wall synthesis activities. The promoter would also be useful in driving the regulated expression of a particular gene product following application of SA or SA analogues to the plant. The TWI1 promoter of FIG 2 could similarly be used to drive the expression of other wound inducible genes substantially homologous to the TWI1 gene in plants other than tomatoes, such as in dicotyledonous and monocotyledonous plants.

It is a further aspect of this invention to provide transformed host cells comprising recombinant DNA encoding a plant GTase in operable linkage with expression signals including promoter and termination sequences which permit expression of said DNA in the

host cell. Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* in procedures known in the art, for example as described in EP-A-0116718 and EP-A0270822.

5 Alternatively, the foreign DNA could be introduced directly into plant cells using electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example, where the recipient plant is monocotyledonous. Any other method  
10 that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would be suitable. This includes species of plants which are not currently capable of genetic transformation.

15 Another aspect of the present invention includes the production of transgenic plants (or parts of them, such as propagating material) containing DNA in accordance with the invention as described above. The constructs  
20 would include a promoter and coding sequence from, for example, the tomato TWI1 gene, or another promoter and coding sequence of plant GTase exhibiting an analogous activation pattern for the purpose of regulated expression of desirable gene products at sites of  
25 attack or following elicitor application. Further, transgenic plants containing the TWI1 coding sequence, other sequence encoding a homologous plant GTase, or fragments thereof, in sense or antisense orientation, under the control of constitutive, developmental or  
30 tissue-specific promoters for the purpose of altering the natural levels of GTase activity are also within the scope of this invention.

Transgene constructs are produced preferably using available promoters and terminator sequences from standard *E. coli* cloning vectors in combination with a GTase coding sequence (such as FIGS. 1, 2, 3 or 4).  
5 Constructs can either then be cloned into other *E. coli* vectors containing plant selectable marker genes, either to be used directly for particle bombardment transformation, or for *Agrobacterium*-mediated transformation when a binary vector will be used.

10 One of the objects of this invention is activation of specific enzyme activity, namely GTase. The activation of GTase by transfer of sequences encoding GTase to other species will not necessarily be species-  
15 dependent. For down-regulation strategies however, it would be a preferred method to use the homologous gene from the target species to enable an efficient antisense effect.

20 It is another aspect of the invention to provide a method for improving the resistance of plants to a very broad spectrum of pests and pathogens by regulating the levels of key signalling intermediates and thus increasing the natural defence responses of plants to  
25 any challenge. In particular, this invention will benefit crops growing in the field and benefit post-harvest care and protection of plant products. For instance, increased basal levels of an unconjugated intermediate in GTase antisense plants could induce a  
30 "resistant state". In ethylene-mediated senescence, this invention seeks to improve the shelf-life/vase-life of products, whether fruit, vegetables, cut flowers, leaves, pot plants etc. This invention also teaches a method of controlling the senescent process,

i.e. inducing ripening at a particular time in response to a spray or changed condition.

5 The following non-limiting examples are provided as an illustration of the usefulness of the above-described invention, wherein reference is made to the following figures:

FIG. 1 The cDNA sequence for the GTase encoded by the TWI1 gene, isolated from tomato.

10 FIG. 2 The 5'upstream region for the TWI1 gene up to the start codon and including the promoter region.

FIG. 3 Amino acid (glucosyl transferase protein) sequence of the TWI1 gene.

15 FIG. 4 Nucleic acid sequence for a homologous GTase enzyme isolated from tobacco.

FIG. 5 Nucleic acid sequence for a homologous GTase enzyme isolated from rice.

20 FIG. 6 Reference gel demonstrating TWI1 expression during stages of tomato fruit development. RNA extracted from tomato fruits at different developmental stages and subjected to Northern Blotting and probed with TWI1 cDNA are shown. Lane 1: immature green fruit; Lane 2: mature green fruit; Lane 3:  
25 breaker stage; Lane 4: pink-ripe; and Lane 5: red-ripe fruit.

30 FIG. 7 Reference gel showing the expression of the proteinase inhibitor (pin)2 gene and ethylene-forming enzyme (ACO) in transgenic tomato lines expressing a TWI1 antisense gene prevents wound-induced pin gene expression and prolongs ACO expression.

FIG. 8 Reference gel electrophoresis demonstrating the accumulation of TWI1 mRNA (GTase) during wounding and elicitor treatment in tomato

plants. Each lane of gel is described in full in Example 2.

5       FIG. 9   Reference gel demonstrating a time-course of TWI1 mRNA accumulation by wounding and salicylic acid (2 mM) treatment in tomato. Each lane is described in full in Example 3.

      FIG. 10   Reference gel showing that wound-induced TWI1 expression is SA-independent.

10       FIG. 11   Reference gel demonstrating local and long range expression of TWI1 on wounding tomato plant leaves.

      FIG. 12   Reference gel illustrating the effect of anti-sense suppression of ACO expression on wound induced pin-2 expression by comparing the levels of transcript accumulation in wounded transformed and wild-type 21 day old tomato plants.

      FIG. 13   Reference gel demonstrating wound-induced pin gene expression can be inhibited by norbornadiene.

20       FIG. 14   Reference gel demonstrating that aspirin inhibits wound induced pin2 gene expression. Details of each lane are given in Example 6.

      FIG. 15   Reference gels demonstrating TWI1 mRNA accumulation in cotyledons of tomato plants injected with either intercellular fluid containing the Avr9 avirulence protein from *Cladosporium fulvum* (IF9) or water. Tomato plants carrying the Cf9 resistance gene (Cf9) or without the resistance gene (Cf0) were used.

25       FIG. 16   Tobacco TWI1 expression and salicylate accumulation in response to TMV infection in tobacco NN plants. The reference gels show the accumulation of the tobacco TWI1 homologue mRNA in TMV-infected wild-type tobacco plants, but no accumulation in mock (water) inoculated plants, nor in plants transgenic for nahG salicylate hydroxylase gene. The time course

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indicates time after transfer of plants from 30°C to 24°C at which point the resistance response is initiated.

5       FIG. 17   Histograms indicating the levels of the free and conjugated salicylate present in leaves of the same plants at the time-points (hours) indicated on the x-axis. Significant SA accumulation only occurs in wild-type NN tobacco, but not NN+ NahG tobacco.

10       FIG. 18   Photographs showing the effects on tobacco leaves of injection of harpin (HrpN) protein into leaves of plants which are wild-type (WT) or transgenic for over-expression of the tobacco TWI1 homologue (OVER) or antisensed for the GTase (ANTI).

15       FIG. 19   Reference gel showing tobacco acidic chitinase (PR3A) accumulation in water- or harpin-treated tobacco leaves at either 1, 2 or 3 days after injections, and in healthy (H) leaves. The plants are either wild-type (WT) or transgenic for over-expression (OVER) or antisensed (ANTI) of the tobacco GTase gene.

20

#### EXAMPLE 1

The TWI1 (tomato wound-inducible) gene was first identified and analyzed as a partial cDNA from a differential screen of a tomato-wounded-leaf cDNA library.

25       Using the partial cDNA as a probe in Northern analyses TWI1 mRNA was confirmed as wound-inducible, with transcripts detectable by 15 minutes after injury to the leaves. Expression of the gene corresponding to TWI1 was also found to be developmentally-regulated. 30       Whilst not expressed in unwounded leaves of a young tomato plant, TWI1 mRNA became abundant in older yellow leaves and was also found at high levels in red-ripe tomato fruit (FIG 6). The pattern of induction of TWI1

by elicitors of other wound responsive genes was also analyzed. The TWI1 was observed to be induced by plant cell wall fragments and salicylic acid, suggesting at the time (P J O'Donnell, 1995, Doctoral Thesis from  
5 Leeds University) TWI1 belonged to the family of defence-related proteins, know as the pathogenesis-related (PR) proteins, all of which are induced by salicylic acid and some of which are also induced by mechanical injury.

10 When a full-length cDNA of TWI1 was obtained and sequenced, homology was found to a large family of previously identified sequences encoding glucosyl transferases. The closest homology to existing  
15 sequences was observed to be that of Mesculenta Crantz cDNAs, mecgt1, encoding a UDP-glucose glucosyl transferase (54.3%) and mecgt5 encoding a UTP-glucose glucosyl transferase (52.2%). These have been identified as transferases involved in glucosylation of  
20 secondary metabolites. High homology was also found to a glucosyl transferase of Zea mays, involved in the glucosylation of IAA (52.8%) and to a ripening-related glucosyl transferase of tomato (ERT1B) known to glucosylate secondary metabolites.

25 The standard approach to identifying function is to use an antisense strategy, in which a transgene is constructed which expresses the gene of interest in antisense orientation, thereby leading to  
30 constitutively negligible levels of the gene product. The phenotype of the plants can then be analyzed to determine the effects of knocking out the expression of the gene. Using this antisense technology, tomato plants were transformed with TWI1 coding sequence in

antisense orientation whose expression was  
constitutively driven by a Cauliflower Mosaic Virus  
(35S) promoter. A 480bp fragment from the 5' end of  
the TWI1 cDNA clone was produced by Polymerase Chain  
5 Reaction using the following primers:

5' primer - TCTTTCCTCTAGAATGCAAGGTC  
incorporating a XbaI restriction site  
10 3' primer - GTTCAGGTACCGATGACACATTC  
incorporating a KpnI restriction site

When digested with XbaI and KpnI, a 461 bp fragment was  
15 produced. This was sub-cloned into XbaI/KpnI digested  
site of the binary vector pJR1Ri, giving a construct  
with the TWI1 fragment in the antisense orientation.  
After transformation into *E. coli*, the plasmid was  
transferred into the *Agrobacterium* strain LBA4404, by  
20 triparental mating. Cultures were then selectively  
grown up of *Agrobacterium* containing the plasmid. This  
was then used to transform tomato plants via  
*Agrobacterium*. Selection of potential transformed  
plants was on the basis of resistance to kanamycin.  
25 Regenerated plants were studied by RNA analysis to  
investigate the effect of the TWI1 antisense transgene.

It was discovered in three independently transformed  
primary transformants that the response to injury had  
30 changed. The standard wound-response gene marker,  
proteinase inhibitor was not expressed, whereas the  
gene encoding ethylene-forming enzyme (ACO), normally  
expressed transiently was not down-regulated and wound  
ethylene levels were maintained at high levels (FIG 7).

35 The revelation that TWI1 was wound-inducible in no way  
implicates the gene product in a regulatory role, quite

the opposite. This gene is responding to wound-induced signals. The fact the TWI1 gene shared homology to those encoding known glucosyl transferases would not implicate the gene product in a regulatory role, since  
5 many glucosyl transferases merely glucosylate secondary metabolites such as ERT1B [14].

#### EXAMPLE 2

Accumulation of TWI1 mRNA by mechanical wounding and  
10 elicitor treatments was assessed. Results can be seen in FIG 8. Wounding was carried out by crushing the terminal leaflets of 21 day old tomato plants (*Lycopersicon esculentum* Mil.) cultivar Moneymaker, with a pair of tweezers. For all elicitor treatments  
15 21 day old plants were excised at the base of the stem and incubated for 30 minutes in the various treatments, at the stated concentrations. After 30 minutes in the elicitor, the plants were transferred into water for the remainder of the incubation period. For all  
20 treatments, plants were maintained under constant light at 22°C. Leaf material was harvested at 1 hour after wounding/treatment in each case, and total RNA was then extracted. 10 µg of total RNA from each sample was separated by agarose gel electrophoresis in gels  
25 containing 7% formaldehyde, blotted onto Hybond-N membrane and probed with P<sup>32</sup> labelled TWI1 cDNA. In FIG. 8 the results are demonstrated, with the labels being: 1) healthy leaf; 2) wounded leaf; 3) H<sub>2</sub>O; 4) jasmonic acid (100 µM); 5) systemin (100 nM); 6)  
30 salicylic acid (2nM); 7) aspirin (2mM); 8) benzoic acid (2mM); 9) 3,4 di-OH benzoic acid (2 mM); 10) 2,6 di-OH benzoic acid (2mM); 11) DCINA (1mM). The filter was exposed to film for 3 days. Equal loading was confirmed by re-hybridising the stripped blot with a <sup>32</sup>P  
35 labelled ribosomal RNA probe.

EXAMPLE 3: Time Course of Expression of TWI1 and  
Response to Wounding And Salicylic Acid

5       FIG. 9 is a Northern analysis comparing the time-course  
of induction of GTase and PR1a (PR= pathogenesis-  
related) gene expression following application of SA to  
the tomato plants through the transpiration stream.  
The increase in steady-state levels of the GTase  
10       transcripts is very rapid when compared to those of  
PR1a, and become detectable within 10-15 minutes of  
treatment. To our knowledge, this is the fastest SA-  
responsive gene so far identified, since the kinetics  
of up-regulation of SAR genes in tobacco and tomato are  
all known to be comparable to that of the PR1a shown in  
15       FIG. 9.

The time-course of TWI1 mRNA accumulation by wounding  
and salicylic acid (2mM) treatment is specifically  
demonstrated in FIG. 9, including the induction of PR1  
20       by SA treatment. 21 day old tomato plants were  
wounded, or excised at the base of the stem and  
incubated with 2 mM salicylic acid, as described in  
Example 2. Leaf material was harvested at each time  
point shown, and total RNA extracted. 10 µg of total  
25       RNA was fractionated through an agarose gel containing  
7% formaldehyde, blotted onto Hybond-N membrane and  
probed with <sup>32</sup>P labelled TWI1 cDNA, or PR1 cDNA. Time  
shown in FIG. 9 is in hours after wound/SA application.  
the filters was exposed to film for 24 hours (TWI1  
30       filters) or 7 days (PR1 filters).

To assess whether SA might be a wound-induced signal  
which induced TWI1 expression, we also wounded leaves  
of transgenic tomato plants harbouring the salicylate

hydroxylase gene (NahG), which presents SA accumulation. As shown in FIG 10, the response of TWI1 to wounding in NahG tomato plants is not significantly different from the response in wild-type plants, indicating that wound induction of this gene is SA-independent.

EXAMPLE 4: (Local and Systemic Wound Induction of TWI1 mRNA)

FIG. 11 compares the timing of wound-induction GTase expression in the leaf that is damaged and in the systemically responding undamaged leaf of the plant.

In FIG. 11, mechanical wounding was carried out on the terminal leaflets the first leaf of 21 day old tomato plants, as described in Example 2. Leaf material was harvested from wounded leaf (local) and the unwounded leaf 2 (systemic) at the stated times, and total RNA extracted. 10 µg of total RNA was separated through an agarose gel containing 7% formaldehyde, blotted onto Hybond-N membrane and probed with <sup>32</sup>P labelled TWI1 cDNA. Time given is hours after mechanical wound. The hybridised filter was exposed to film for 8 days.

EXAMPLE 5

The effect of antisense suppression of the ethylene - forming enzyme (ACO) expression on wound induced pin-2 expression was assessed by comparing the levels of transcript accumulation in wounded transformed and wild-type plants. 21 day old tomato plants (lycopersicon esculentum Mill) cultivar Alisa Craig, expressing an ACO construct in anti-sense orientation and driven by the 35S CaMV promoter were wounded as discussed in Example 2, above. Leaf material was

harvested at the indicated times, total RNA was  
extracted and analyzed for pin-2 gene expression by  
Northern blot. Control wild type plants were wounded  
and leaf material harvested at 8 hours for Northern  
5 analysis.

We also found that the inhibitory effect of NBD  
(norbornadiene, a competitive inhibitor of ethylene on  
pin2) expression can be overcome by excess exogenous  
10 ethylene, which is consistent with its action as an  
inhibitor (FIG. 12). This strongly suggests that  
ethylene must be present in wound response. Results  
illustrating pin2 gene expression following these  
treatments are shown in FIGS 12 and 13.

15

#### EXAMPLE 6

Aspirin inhibition of pin-2 gene expression can be  
overcome by jasmonic acid and ethylene, but not by JA  
or ethylene alone. Plants were pretreated in water or  
20 ASA (aspirin) for 30 minutes, in gas tight chambers,  
before removal of the plants and incubation in the  
open. ASA pretreated plants were treated with either  
ethylene (100 ppm), JA, JA + 5ppm of ethylene, JA + 10  
ppm ethylene, JA + 50 ppm ethylene or JA + 100ppm  
25 ethylene for 30 minutes in gas tight chambers before  
transfer to the open. Control plants were treated with  
water for the experimental duration. Leaf material was  
harvested at 4 hours post-treatment, total RNA  
extracted and northern analysis performed using the  
30 pin-2 cDNA (FIG 14).

#### EXAMPLE 7

The expression of TWI1 in response to gene-for-gene  
mediated pathogen resistance was assessed using Avr9-

containing extracts to elicit a resistance response in tomato harbouring the Cf9 resistance gene. Elicitor was injected into cotyledons of Cf9 plants or control plants with no Cf9 gene (Cf0) and as a further control, Cf9 cotyledons were injected with water. At various time-points after injection, RNA was extracted and subjected to Northern blotting using the TWI1 cDNA as a probe (FIG 15). Significant induction of TWI1 expression was only detected in Cf9 plants injected with Avr9 elicitor, demonstrating a pathogen-resistance response-specific activation of the GTase.

#### EXAMPLE 8

The expression of the tobacco TWI1 homologue during a pathogen-resistance response was investigated in tobacco plants harbouring the N-resistance gene infected with tobacco mosaic virus (TMV). Wild-type tobacco, or tobacco transformed with a salicylate hydroxylase gene (NahG) which cannot accumulate salicylic acid (SA), were inoculated with TMV or mock-inoculated with water and grown for 2 days at 30°C to permit virus spread. The plants were then transferred to 24°C to initiate the resistance response and RNA, SA and SA conjugates were extracted at various time-points. As shown in FIGS 16 and 17, GTase expression was only induced in wild-type NN tobacco infected with TMV. No GTase expression was observed in the NahG transformants which did not significantly accumulate SA, whereas expression in wild-type plants correlated with the timing of SA production.

#### EXAMPLE 9

The tomato TWI1 cDNA sequence can be used as a heterologous probe to identify the SA GTase from a

range of Solanaceous species as shown by Southern blotting. We have isolated full-length cDNA clones corresponding to the SA GTase of tobacco using the tomato TWI1 sequence as a probe (FIG 4). These two  
5 genes show around 85% identity in primary sequence. Additional GTases have been identified in DNA sequence databases of expressed sequence tags from rice (FIG 5).

EXAMPLE 10 - Production of transgenic plants

10 Plasmid constructs containing the tobacco TWI1 GTase homologue in either sense or antisense orientation were produced using the pJR1Ri vector, placing expression of the sense or antisense genes under the control of the CaMV 35S promoter and nos polyadenylation signal.  
15 These plasmids were transferred to *Agrobacterium tumefaciens* strain LBA4404 by tri-parental matings. Leaf disks from tobacco *Nicotiana tabacum* cv (Samsun NN) were inoculated with the transformed *Agrobacterium* strains and transgenic plants regenerated using  
20 standard protocols.

These plants were used in experiments using the bacterial elicitor, harpin (the *HrpN* gene product from *Erwinia amylovora*). In wild-type tobacco plants, and  
25 plants expressing an antisense GTase gene, harpin injection into leaves caused the formation of necrotic lesions, but such lesions were not observed in plants over-expressing the GTase (sense construct) (FIG 17). RNA was extracted from the injected leaves and the  
30 expression of PR genes assessed by Northern blotting (FIG 18). In wild-type plants, PR3a gene expression peaked at 1 day post-injection and was present at high levels throughout the time-course. In antisense plants, the peak at 1 day was maintained over the whole

time course, whereas in over-expressing plants, PR3a expression was significantly suppressed.

5       These data suggest that a key signal which controls lesion formation and PR gene expression is affected by GTase expression levels.

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CLAIMS

1. A recombinant or isolated DNA as shown or substantially homologous to a nucleotide sequence of FIG 1 or a fragment thereof, wherein said DNA is a gene (TWI1) encoding a glucosyl transferase or an equivalent protein in plants.
2. DNA as claimed in claim 1 encoding for an amino acid sequence of FIG 3.
3. A recombinant or isolated DNA as shown in FIG 4 or a fragment thereof, wherein said DNA codes for a substantial homologue of a tomato TWI1 gene and is isolated from tobacco.
4. A recombinant or isolated DNA as shown in FIG 5 or a fragment thereof, wherein said DNA codes for a substantial homologue of a tomato TWI1 gene and is isolated from rice.
5. DNA as claimed in claim 1, which further comprises a promoter said promoter being 5' to the coding region of the sequence shown in FIG 2.
6. DNA as claimed in any one of claims 1 to 5, wherein the promoter is operatively linked to the TWI1 gene which, when expressed, effects glucosyl transferase levels in tomatoes.
7. DNA as claimed in any one of claims 1 to 6, wherein the TWI1 promoter of FIG 2 is operatively linked to DNA substantially homologous to the TWI1 gene which, when said DNA is expressed, effects glucosyl transferase levels or a similar protein in dicotyledonous plants.

8. DNA as claimed in any one of claims 1 to 6, wherein the TWI1 promoter of FIG 2 is operatively linked to DNA substantially homologous to the TWI1 gene which, when said DNA is expressed, effects glucosyl transferase levels or a similar protein in monocotyledonous plants.

9. DNA as claimed in any one of claims 1 to 8, wherein said DNA encodes for RNA which is antisense to RNA normally found in a plant cells.

10. DNA as claimed in claim 9, wherein the DNA encodes for RNA which is antisense to RNA encoding for glucosyl transferase in tomatoes or its equivalent in other plants.

11. Antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of DNA that is naturally transcribed by the TWI1 gene coding for glucosyl transferase in tomatoes or an equivalent gene and protein in other plants.

12. Antisense nucleic acid as claimed in claim 11, wherein transcription is under the control of a constitutive promoter, such as a Cauliflower Mosaic Virus 35S promoter.

13. Antisense nucleic acid as claimed in claim 11, wherein transcription is under the control of a glucosyl transferase promoter.

14. DNA as claimed in any one of claims 1 to 13, which is in the form of a recombinant vector.

15. DNA as claimed in claim 14, wherein the vector is a cloning vector and comprises one or more selectable markers.

16. A microbial host cell transfected or transformed with a vector as claimed in claim 14 or 15.

5 17. DNA as claimed in any one of claims 1 to 16, which includes a marker sequence which enables a plant transformed with the DNA to be distinguished from plants not so transformed.

10 18. DNA as claimed in claim 17, wherein the marker sequence confers antibiotic or herbicide resistance.

19. A plant cell including DNA as claimed in any one of claims 1 to 18.

15 20. A plant or part of a plant, or propagating material from a plant comprising plant cells transformed as claimed in claim 19.

20 21. A method of altering the signalling pathways in plants by altering the levels of any one of salicylic acid, jasmonic acid or ethylene produced by said plants, said method comprising interfering with a glucosyl transferase or similar protein encoded by an isolated DNA (TWI1) as claimed in any one of claims 1 to 18 or a  
25 fragment thereof.

22. A method as claimed in claim 21, wherein the plants are any one of tobacco, rice or tomato, preferably tomatoes.

30 23. A method of inducing the production of plant defence proteins in plants, said method comprising regulating a glucosyl transferase protein encoded by an isolated DNA (TWI1) as shown in or substantially homologous to a  
35 nucleotide sequence as claimed in any one of claims 1 to 18, said glucosyl transferase altering the signalling

pathways of any one of salicylic acid, jasmonic acid or ethylene.

24. A method as claimed in claim 23, wherein the plant  
5 defence proteins comprise PR proteins and PIN proteins.

25. A method of regulating developmental events in  
plants, wherein said method comprises manipulating levels  
of ethylene produced by a plant, said manipulation  
10 comprising interfering with production of a glucosyl  
transferase gene encoded by an isolated DNA as claimed in  
any one of claims 1 to 18 or a fragment thereof.

26. A method as claimed in claim 25, wherein the  
15 developmental events regulated are plant growth,  
reproduction and senescence.

27. A method for stimulating or improving plant response  
to pathogens, said method comprising altering levels of  
20 salicylic acid produced by a plant in response to a  
pathogen, said method further comprising interfering with  
a glucosyl transferase gene encoded by a DNA as claimed  
in any one of claims 1 to 18 or a fragment thereof.

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1 TCATTTTTTC TTCTTTCCCG ATGATGCTCA AGGTCATATG ATACCTACAC  
51 TTGACATGGC GAACGTTGTC GCTTGTCGTG GTGTTAAAGC CACTATAATC  
101 ACAACACCTC TCAATGAATC TGTTTTCTCT AAAGCTATTG AGAGAAACAA  
151 GCATTTAGGT ATTGAAATTG ATATTCGTTT ACTAAAATTC CCAGCTAAGG  
201 AGAATGATTT GCCTGAAGAT TGTGAGCGTC TTGATCTTGT ACCTTCTGAT  
251 GACAAACTCC CAAACTTCTT AAAAGCTGCG GCTATGATGA AAGATGAATT  
301 TGAGGAGCTT ATTGGAGAAT GTCGCCCTGA TTGTCTTGTT TCTGATATGT  
351 TCCTTCCATG GACTACTGAT AGTGCAGCCA AATTTAGCAT ACCAAGAATT  
401 GTATTCCATG GAACTAGTTA CTTTGCCCTT TGTGTTGGCG ATACGATCAG  
451 GCGTAATAAG CCTTTCAAGA ATGTGTCATC GGATACTGAA ACTTTTGTTG  
501 TACCGGATTT GCCACATGAA ATTAGGCTAA CTAGAACACA GTTGTCTCCG  
551 TTTGAGCAAT CGGATGAAGA GACGGGTATG GCTCCCATGA TTAAAGCTGT  
601 GAGGGAATCG GATGCGAAGA GCTATGGAGT TATATTCAAT AGCTTTTATG  
651 AGCTTGAATC AGATTATGTT GAACATTACA CTAAGGTTGT AGGTAGAAAA  
701 AATTGGGCTA TTGGTCCGCT TTCGCTGTGC AATAGGGATA TTGAAGATAA  
751 AGCGGAAAGA GGGAGGAAAT CATCTATCGA TGAACACGCG TGCTTGAAAT  
801 GGCTTGATTG GAAGAAATCA AGTTCCATTG TTTATGTTG TTTTGGAAGT

FIG. 1(I)

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851 ACAGCAGATT TCACTACAGC ACAGATGCAA GAACTTGCTA TGGGGCTAGA  
901 AGCCTCTGGA CAAGATTTCA TTTGGGTTAT CAGAACAGGG AATGAAGATT  
951 GGCTCCCAGA AGGATTCGAG GAAAGAACAA AAGAAAAAGG TTTAATCATA  
1001 AGAGGATGGG CACCCCAAAG TGTGATTCTT GATCACGAAG CTATTGGAGC  
1051 TTTTGTTACT CATTGTGGAT GGAAGTCGAC ACTGGAAGGA ATATCAGCAG  
1101 GGGTACCAAT GGTGACATGG CCAGTATTTG CGGAACAGTT TTTCAATGAG  
1151 AAGTTGGTGA CTGAGGTAAT GAGAAGTGGA GCTGGTGTTG GTTCTAAGCA  
1201 ATGGAAGAGA ACAGCTAGTG AAGGAGTGAA AAGAGAAGCA ATAGCAAAGG  
1251 CGATAAAGAG AGTAATGGCG AGTGAAGAAA CAGAGGGATT CAGAAGCAGA  
1301 GCAAAAGAGT ACAAAGAAAT GGCAAGAGAA GCTATTGAAG AAGGAGGATC  
1351 ATCTTACAAT GGATGGGCTA CTTTGATACA AGACATAACT TCATATCGTT  
1401 AACTAGTTGA TGCAAAAAAA GAAAAACAT GTGTGTTTCT ATATTCTGTC  
1451 TTCTGTTTTG CTGATTTGAT CATATTACGT ACTTCTTCAT GATAATTAAT  
1501 GACATCAATA GAATCCAAGA TCAATCATCT CGAAATTCAA CGTTAAAATA  
1551 TTTCGACATT TGAATAATAC ATCGACTTAA AATGGAAAAA AAAAAAAAAA  
1601 AAAAAAAAAA AAAAAAAAAA AAAA

FIG. 1(II)

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AAGCTTACAAGATAGTGTTCATGTAGGCCGAAAAAGATAGAAAATTATTAA  
TAAATTAAATTTAAGAGGTAATATAACCTTATTATAATATAAATGTGTAT  
CTAAAATTTCTGACATAAATCTAGGGAATAGTTATACATTATTCTTTATT  
ATTATTATTGAGTCGTCAAAAAATATTATTAGAATTTATGAGCTAATACA  
TATTTAATTTTATAATGTAAATATATTTTTTTTAAAAATTTACCGACTTCA  
ATAGAACCCACGAACCTTATCTATATCCGCCTCGTGACCACCACCTTCT  
CAAGTATTCGCCAAAATCAAATGGCAATTACCGGTTCCCTACTGCAATAA  
TTTAGCAGCTAATGAACAAAATGCATCTTGTCATCTTCTAGATGATTTGT  
ACTTCTTTCTGCTTAATAATAATCGTTGACCGTTGATTTAACATAAAAAG  
ACAAATGACTCGAAATAATGATTAATAATAATAAATGATAAAGGTACTAT  
AATACTGTACTAAGTACTAGCATT-----  
TGTATTTTCGTCTTTGAGCAAAGCACTGTCGTTAAATTAACCTAAATAAT  
AAAAGAAAAGTTTGTGTTTCAATTGACCTGTGAGGGGAGGGCATATGTCA  
CCTGTAAAGTTACATCTGCCAAGAAATTCATACGCTGGTCCCCGCTATT  
GCCGGTTTCCTTCATTGACAAGGCCAGTTATTTTGTGAGATTGAATTTATT  
TCACCTCCGATTATTAGATATTTATTAAAATCGATATATGTTTAGTCATG  
ATTTTATATATAAAATAGTTATGATCACGAAATATTTGATCTTAAACTA  
ATAAACCTTGATGAGTTGTTTAGGGTTTGTCTGTTAAATCAACCACGTG  
AGTGCTTACGTGACTTCTTTACGTCCAACCTGAAATAACAAGTTAATCTC  
TTATGCAACTTCAACGAAAGTCTTCAAACAATTTCGACGTATATATAACGT  
GACACTAATGCAATTACAACTCAAAAAGTATTTACTCCTCTTTGTTTCAT  
TCAAGCATTTCCACAA ATG

FIG. 2

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1 MGELHFFFFP DDAQHMIPT LDMANVVACR GVKATIITTP LNESVFSKAI  
51 ERNKHLGIEI DIRLLKFPK ENDLPEDCER LDLVPSDDKL PNFLKAAAMM  
101 KDEFEEELIGE CRPDCLVSDM FLPWTTDSAA KFSIPRIVFH GTSYFALCVG  
151 DTIRRNKPFK NVSSDTETFV VPDLPEIIRL TRTOLSPFEO SDEETGMAPM  
201 IKAVRESDAK SYGVIFNSFY ELES DYVEHY TKVVGRKNWA IGPLSLCNRD  
251 IEDKAERGRK SSIDEHACLK WLDSKKSSSI VYVCFGSTAD FTTAOMOELA  
301 MGLEASQDF IWVIRTGNED WLPEGFEERT KEKGLIIRGW APOSVILDHE  
351 AIGAFVTHCG WNSTLEGISA GVPMTWPVF AEOFFNEKLV TEVMRSGAGV  
401 GSKQWKRTAS EGVKREAIK AIKRVMASEE TEGFRSRAKE YKEMAREAIE  
451 EGGSSYNGWA TLIQDITSYR

FIG. 3

1 AAGAACTGAA AACAACCACA CGTCTTTACT TTTCTTTCTG CTTTCTGATA  
51 CTAAACTACA TTTTCTTTTC TTTCATTCAA ACATTTTCAC AAATGGGTCA  
101 GCTCCATTTT TTCTTCTTTC CTGTGATGGC TCATGGCCAC ATGATTCCTA  
151 CGCTAGACAT GGCCAAGCTC GTTGCTTCAC GTGGAGTTAA GGCCACTATA  
201 ATCACAACCC CACTCAATGA ATCCGTTTTTC TCCAAATCTA TTCAAAGAAA  
251 CAAGCATTTG GGTATCGAAA TCGAAATCCG TTTGATCAAA TTCCCAGCTG  
301 TTGAAAATGG CTTACCTGAA GAATGCGAGC GCCTCGATCT CATCCCTTCA  
351 GATGATAAGC TCCCAAACCTT CTTCAAAGCT GTAGCTATGA TGCAAGAACC  
401 ACTAGAACAG CTTATTGAAG AATGTCGACC CAATTGTCTT GTTTCTGATA  
451 TGTTCCTTCC TTGGACTACT GATACTGCAG CCAAATTTAA CATGCCAAGA  
501 ATAGTTTTTC ATGGCACAAG CTTGTTTGCT CTTTGTGTGCG AGAATAGCAT  
551 CAGGCTAAAT AAGCCTTCA AGAATGTCTC CTCTGATTCT GAAACTTTTG  
601 TTGTACCGAA TGTGCCTCAC GAAaTAAaTG ACCAGACCCA GTTG

FIG. 4

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1 CTTTCCCGGC CGCCGAGGCG CNTNANCCGG AGGGGTGCGA GAGGGTGGAC  
51 CACGTCCCCT CGCCGGACAT GGTGCCGAGC TTCTTCGACG CCGCCATGCA  
101 GTTCGGCGAC GCAGTGGCGC ANACTNCNGG CGCCTCACGG GGCCGCGCCG  
151 GCTGAGCTGC CTCATCGCCG GGATATCTCA CACGTGGGCG CACGTCCTGG  
201 CGCGCNACTC GGCGCTCCGT GCTTCATCTT CCACGGTTTC TGC GCGTTCT  
251 CCCTGCTCTG CTGCNAGTAC CTGCACGCGC ACAGGCCGCA CGAGGCGGTC  
301 TCCTCGCCGG ACGAGCTCTT TGACGTCCCT GTCCTGCCGN CTTTCGAGTT  
351 CAGG

FIG. 5

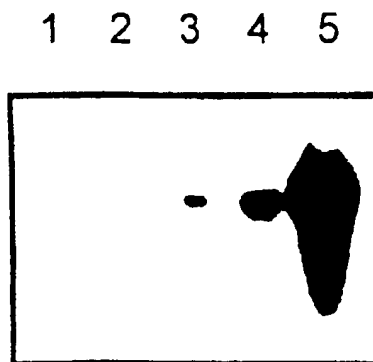


FIG. 6

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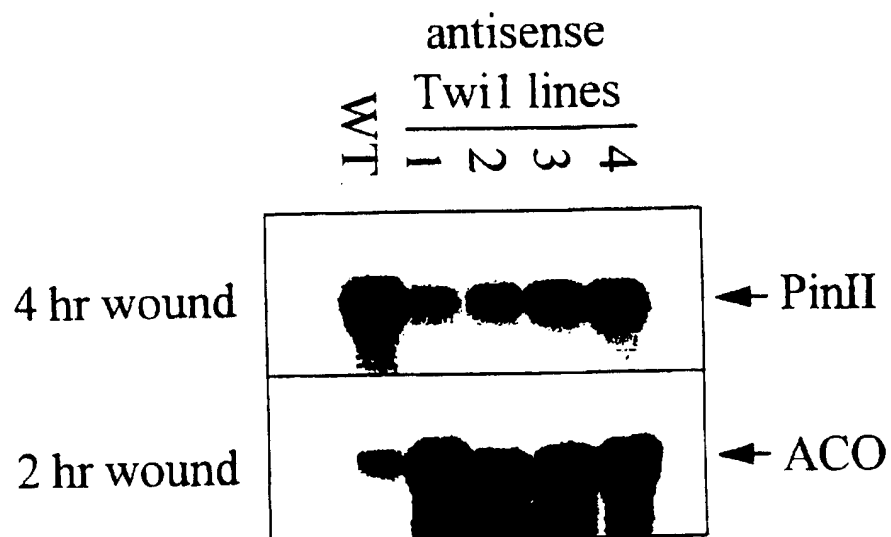


FIG. 7

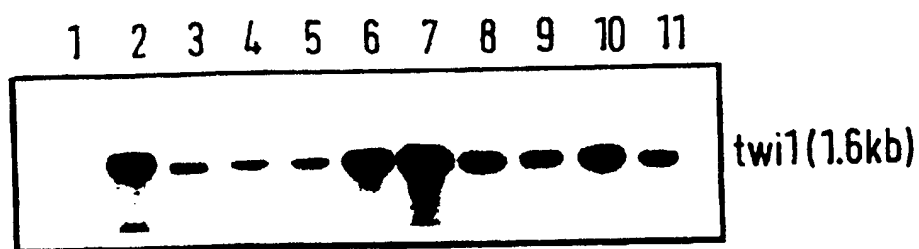


FIG. 8

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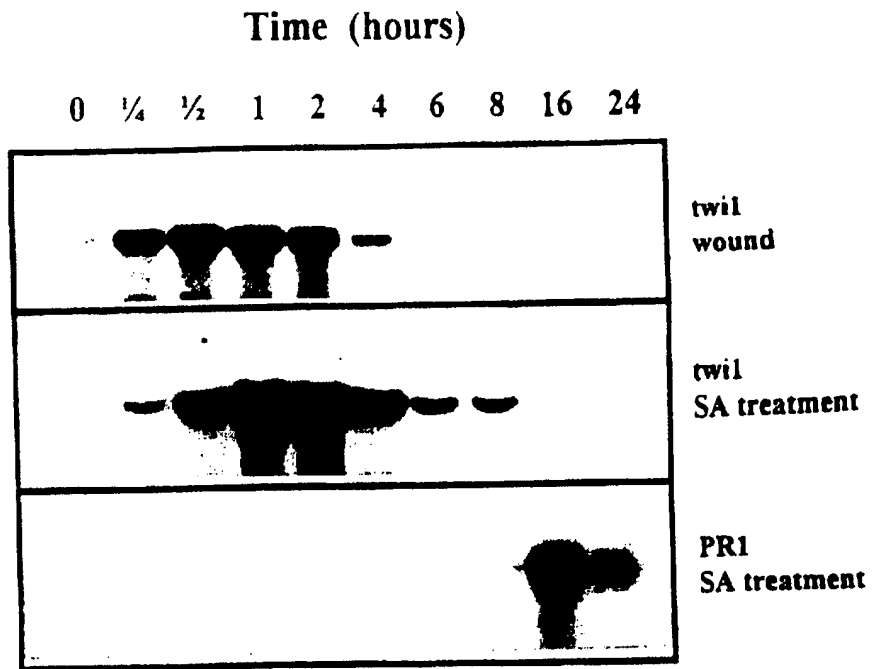


FIG. 9

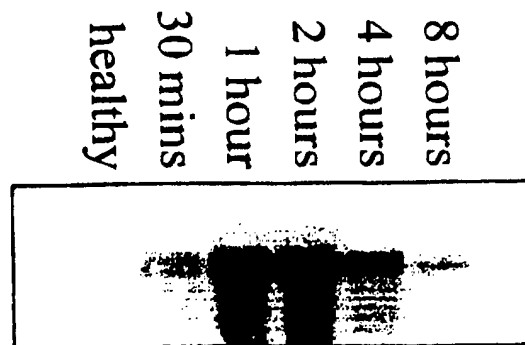


FIG. 10

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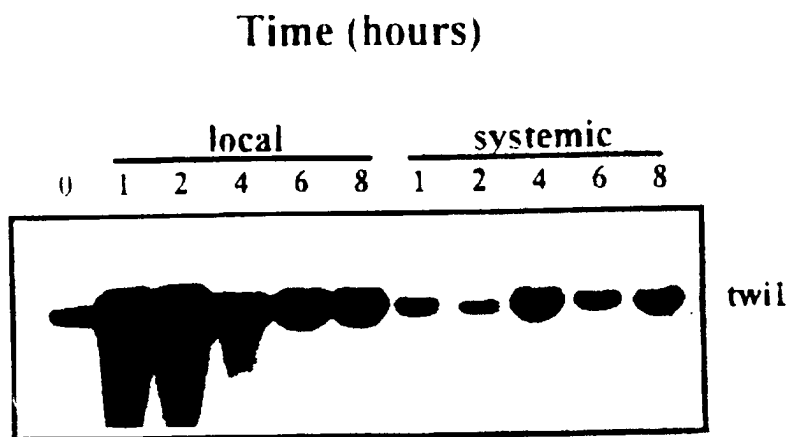


FIG. 11

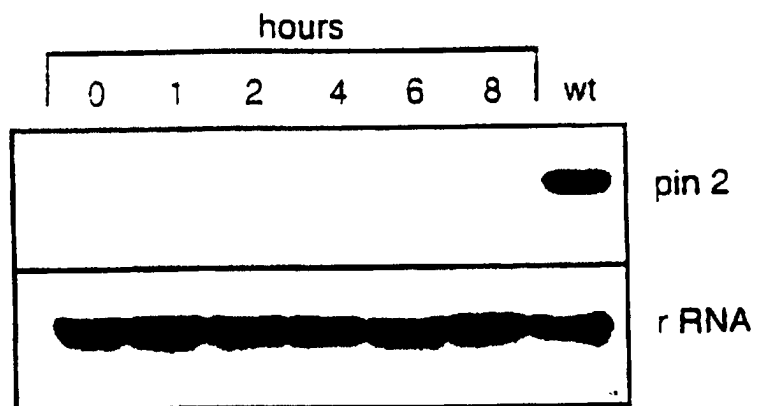


FIG. 12

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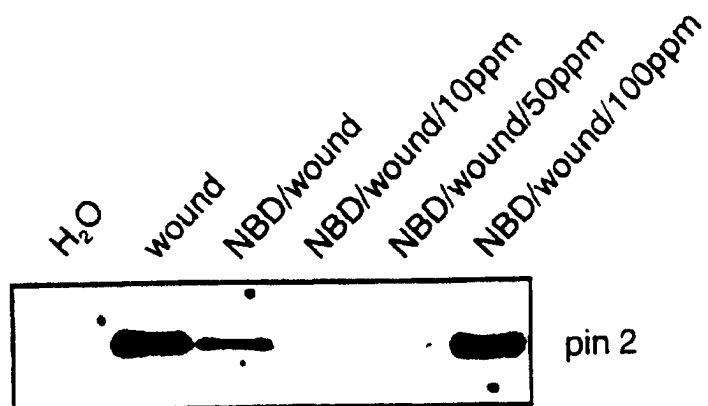


FIG. 13

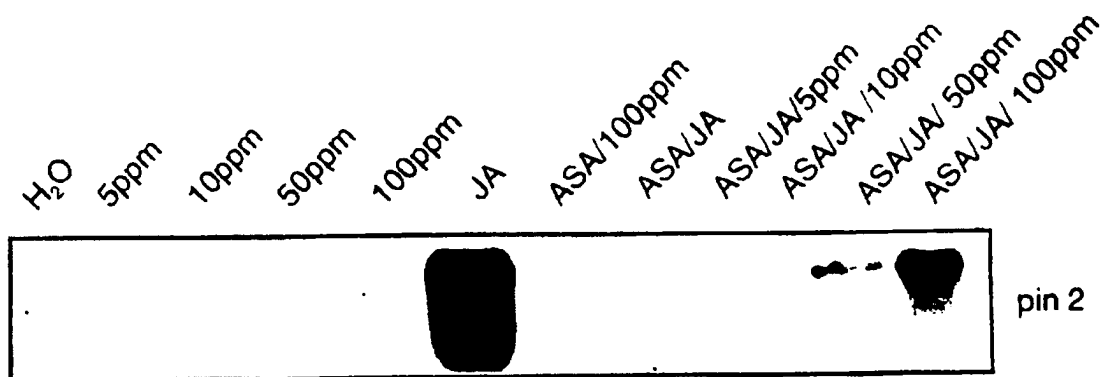


FIG. 14

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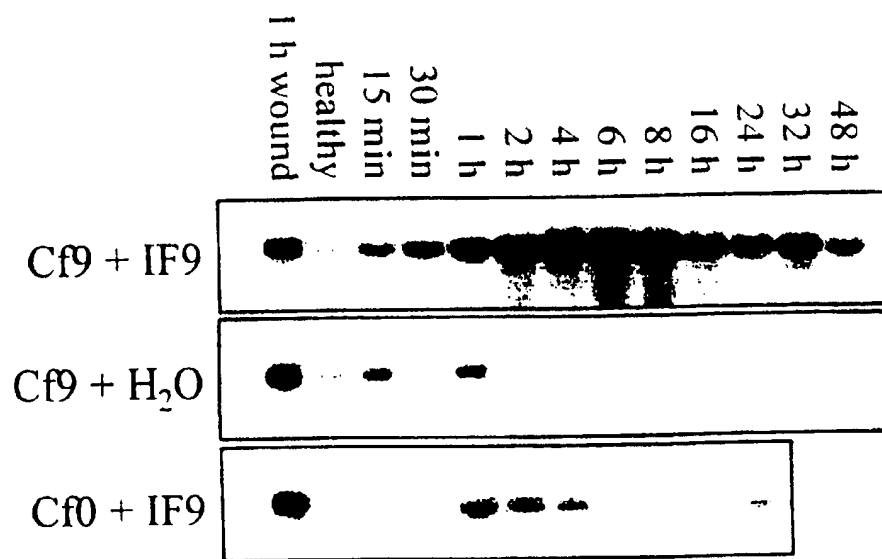


FIG. 15

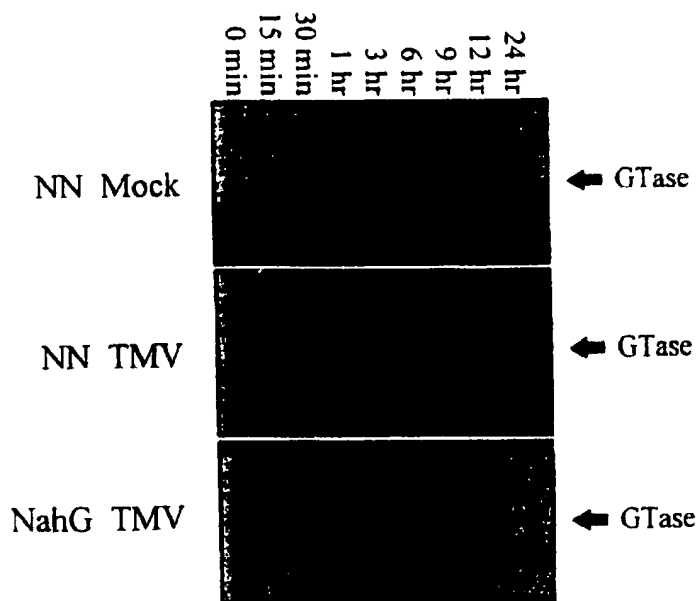


FIG. 16

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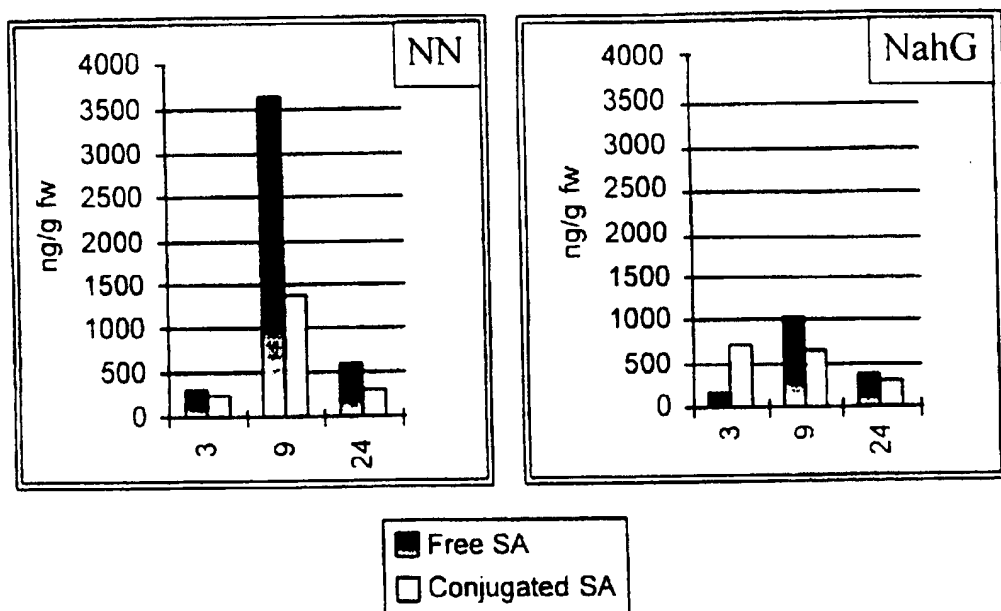


FIG. 17

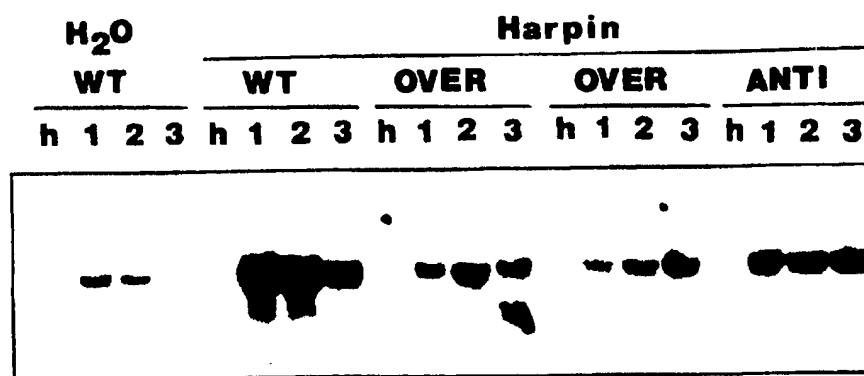
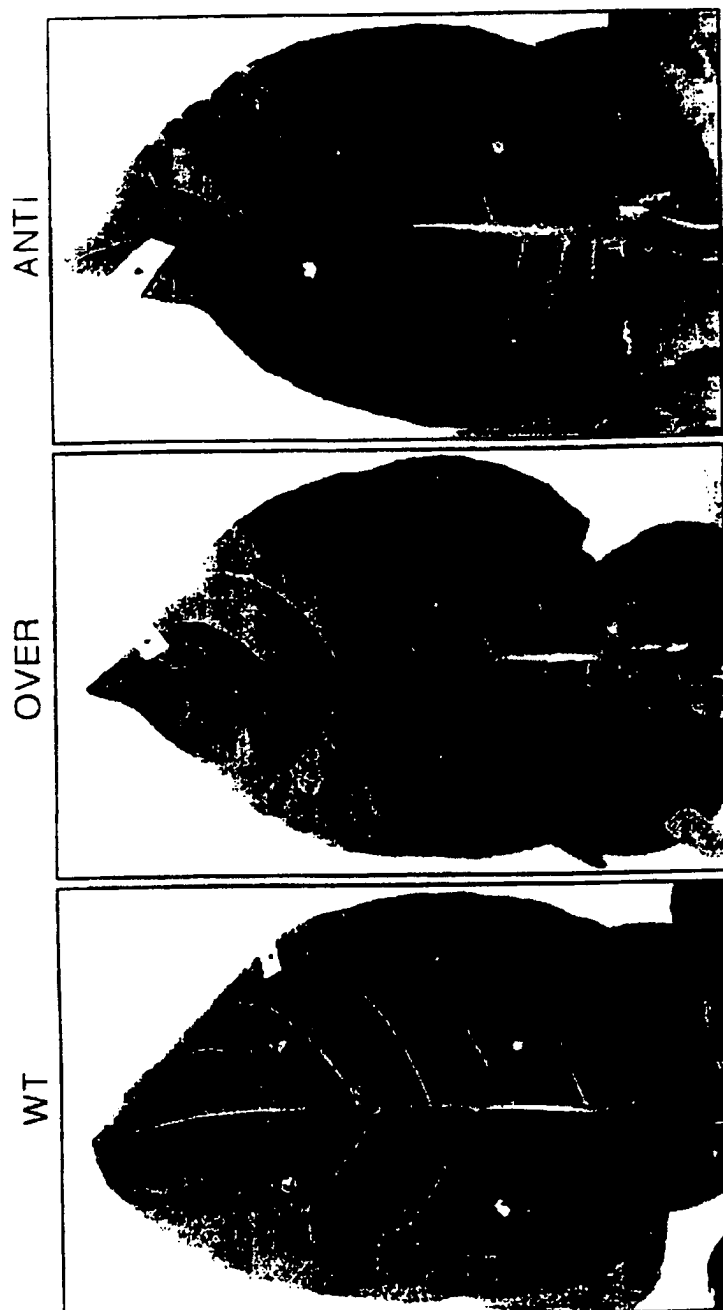


FIG. 19

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FIG. 18



# INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/GB 97/01473

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/54 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRUESDALE M.R.: "L.esculentum twi1 mRNA" EMBL SEQUENCE DATABASE, RELEASE 45, 31-AUG-1995, ACCESSION NO. X85138, XP002043235 see sequence	1,2, 9-11,14, 16
X	--- SASAKI, T., ET AL.: "Rice cDNA, partial sequence (S1943_1A)" EMBL SEQUENCE DATABASE, REL.41 13-NOV-1994, ACCESSION NO. D40163, XP002043236 see sequence --- -/-	4



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

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Date of the actual completion of the international search

10 October 1997

Date of mailing of the international search report

24.10.97

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01473

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>LEE, HYUNG-IL ET AL: "Biosynthesis and metabolism of salicylic acid"</p> <p>PROC. NATL. ACAD. SCI. U. S. A. (1995), 92(10), 4076-9 , XP002043238</p> <p>see the whole document</p>	1-27
A	<p>DEMPSEY D A ET AL: "SIGNALS IN PLANT-DISEASE RESISTANCE"</p> <p>BULLETIN DE L INSTITUT PASTEUR, (JUL/SEP 1995) VOL. 93, NO. 3, PP. 167-186. , XP002043239</p> <p>see the whole document</p>	1-27
A	<p>SEO S ET AL: "INDUCTION OF SALICYLIC-ACID BETA-GLUCOSIDASE IN TOBACCO-LEAVES BY EXOGENOUS SALICYLIC-ACID"</p> <p>PLANT AND CELL PHYSIOLOGY, (APR 1995) VOL. 36, NO. 3, PP. 447-453. , XP002043240</p> <p>see the whole document</p>	1-27
A	<p>YALPANI, NASSER ET AL: "Partial purification and properties of an inducible uridine 5'-diphosphate-glucose: salicylic acid glucosyltransferase from oat roots"</p> <p>PLANT PHYSIOL. (1992), 100(1), 457-63 , XP002043241</p> <p>see the whole document</p>	1
A	<p>ENYEDI, ALEXANDER J. ET AL: "Induction of UDP-glucose: salicylic acid glucosyltransferase activity in tobacco mosaic virus-inoculated tobacco (Nicotiana tabacum) leaves"</p> <p>PLANT PHYSIOL. (1993), 101(4), 1375-80 , XP002043242</p> <p>see the whole document</p>	3

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01473

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SILVERMAN, PAUL ET AL: "Salicylic acid in rice. Biosynthesis, conjugation, and possible role" PLANT PHYSIOL. (1995), 108(2), 633-9 , XP002043243 see the whole document ---	4
A	ENYEDI A J ET AL: "LOCALIZATION, CONJUGATION, AND FUNCTION OF SALICYLIC-ACID IN TOBACCO DURING THE HYPERSENSITIVE REACTION TO TOBACCO MOSAIC-VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (15 MAR 1992) VOL. 89, NO. 6, PP. 2480-2484. , XP002043244 see the whole document ---	1
A	WO 95 12304 A (UNIV RUTGERS) 11 May 1995  see page 11, line 16 - page 12, line 4 ---	21,23, 25,27
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Information on patent family members

International Application No

PCT/GB 97/01473

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		JP 8507923 T	27-08-96
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